Azacytidine targeting SARS-CoV-2 viral RNA as a potential treatment for COVID-19

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Running title: Azacytidine as potential COVID-19 treatment

Abstract

The COVID-19 pandemic is a global health disaster. Moreover, emerging mutated virus strains present an even greater challenge for existing vaccines and medications. One possible solution is to design drugs based on the properties of virus epigenome, which are more common among coronaviruses.

Here, we reported an FDA-approved drug for myelodysplastic syndrome, azacytidine (5Aza), limited virus infection and protected mice against SARS-CoV-2. We demonstrated that this antiviral effect is related to 5Aza incorporation into viral RNA, which disrupt m5C RNA methylation modification profile. This work suggests that targeting viral epigenomes is a viable therapeutic strategy, potentially opening new pathways for treating COVID-19.

The COVID-19 pandemic is a global health disaster. Moreover, emerging mutated virus strains present an even greater challenge for existing vaccines and medications. One possible solution is to design drugs based on the properties of virus epigenome, which are more common among coronaviruses.

As an FDA-approved drug for myelodysplastic syndrome, 5Aza is a structural analog of cytidine (Fig.S1). It exhibits antiviral effects against several viruses. However, it is unknown whether this extends to SARS-CoV-2.

Here, we demonstrated that 5Aza shows potent antiviral activity in Vero E6 cells, with half-maximal inhibitory concentration (IC_{50}) = 6.99 μ M and selective index (SI) = 20.41 (Fig.1a). The viral titer in cell supernatants detection (Fig.1b) and indirect immunofluorescence assay (IFA) against viral N protein (Fig.1c) revealed 5Aza restricted SARS-CoV-2 infection in a dose-dependent manner. Time-of-drug-addition assay (Fig.S2a) showed that 5Aza functioned after virus entry (Fig.S2b, c, and d). The virus replication is still 50% suppressed when 5Aza at 32 μ M is added 24 hours after infection (Fig. S3).

We then used 5Aza for *in vivo* antiviral evaluation in BALB/c mice infected with a mouse-adapted SARS-CoV-2 (MA-SARS-CoV-2) that exhibits high infectivity in mice. In clinical use, the recommended starting dose of 5Aza is 75 mg/m² (equals to ~2mg/kg) in treating MDS. Therefore, mice were intranasally challenged with 2×10^3 plaque-forming units (PFU) of MA-SARS-CoV-2, followed by intraperitoneal administration of 5Aza at 2 mg/kg body weight

69 (equal to 6 mg/m² surface area) at 1 d post infection (dpi), once daily for seven
70 consecutive doses. As shown in Fig.1d, at 5 dpi, mice in 5Aza-treated group
71 started to recovery body weight, while mice body weight in saline-treated group
72 were still decreasing and 5/6 of mice lost more than 25% of body weight.
73 In accordance with this, 83.33% (5/6) of saline-treated animals became
74 moribund (defined as 25% loss of body mass) compared with 16.67% (1/6) in
75 5Aza-treated group. Besides, the viral RNA copy number and titers in the
76 lungs of 5Aza-treated mice also showed a significant decrease (Fig.1f).
77 Moreover, histological examination revealed remarkable amelioration of lung
78 damage at 4 dpi in the 5Aza group (Fig.S4c). In contrast, the saline group
79 exhibited massive alveolar space mononuclear cell infiltration, moderately
80 severe bronchiolar epithelial cell death, and intra-endothelium and
81 perivascular infiltration of pulmonary blood vessels (Fig.S4b). The RNA-seq of
82 lungs also demonstrated that the 5Aza rescued most downregulated genes
83 with virus infection (Fig.S5), further demonstrated that 2 mg/kg 5Aza treatment
84 protected against SARS-CoV-2 attack. Based on the dose used in mice is
85 equivalent to that in treating MDS in humans, it would be encouraging to
86 extend to human COVID therapy.

87 As an RNA analog with OH-group on ribose 2' carbon (2' C) (Fig.S1a),
88 5Aza could theoretically incorporate into RNAs. High-resolution mass
89 spectrometry analysis showed that RNA containing 5Aza increased ~40-fold

with short-term 5Aza treatment (Fig.S6). We took advantage of 5Aza stability in bisulfite treatment to develop a new method (5Aza-BSseq) that identifies the location where 5Aza is incorporated (Fig.S7). Notably, 5Aza-BSseq results also suggested efficient azacytidine incorporation into viral RNA (Fig.1g).

After deoxidized conversion to decitabine and incorporation into DNA, 5Aza causes endogenous retrovirus (ERV) DNA hypomethylation, which activates retroviral RNA transcription and triggers type I interferon response. In this study, decitabine was five times less efficient than 5Aza in inhibiting viral replication (Fig.S8). Additionally, 5Aza did not significantly increase ERV expression (Fig.S9). Therefore, 5Aza incorporation into RNA might be linked to its inhibitory effects on viral replication.

We further explored the possible antiviral mechanism of 5Aza incorporation into viral RNA. Previous research showed that 5Aza incorporation enhanced lethal mutagenesis on influenza virus. However, we found consistent mutations and comparable mutation rates in the viral RNA propagated in 5Aza-treated or control cells (Supplementary Table 1), excluding the role of 5Aza-induced lethal mutagenesis in anti-SARS-CoV-2. Considering that 5Aza sequesters tRNA methyltransferase to inhibit cytosine-C5 methylation (m^5C) in tRNA¹ (the reaction principle is exhibited in Fig.S10), we further explored whether 5Aza incorporation affects viral RNA methylation. We used nanopore direct RNA sequencing to avoid false-positive

methylation sites caused by unconverted 5Aza, as assessed with bisulfite sequencing. The nanopore m⁵C identification algorithm, trained by m⁵C control data as well as many datasets, was used for data analysis. High-confidence m⁵C sites in 5Aza-treated viral RNA decreased significantly by 40% (false positive < 0.05) (Fig.1h). We further validated these m⁵C sites using the optimized RNA-BSseq and strict criteria (Fig.S11 and S12). All m⁵C sites identified through nanopore sequencing were also present in RNA-BSseq (Fig.S13).

The primary writers for m⁵C on mRNAs have been proposed to be NSUN2 and DNMT2, which are demonstrated to contribute to m⁵C methylation on HIV-1 RNA and thus facilitate virus infection^{2,3}. Here, we found that overexpression of DNMT2 and NSUN2, significantly promoted the SARS-CoV-2 replication (Fig.S14), implying both of DNMT2 and NSUN2 are responsible for the m⁵C methylation of SARS-CoV-2 RNA. The immunoprecipitation (IP) assay showed that DNMT2 and NSUN2 could bind to SARS-CoV-2 RNA (Fig. 1i). Notably, DNMT2 IPed more RNAs in the presence of 5Aza. As RNA methylation occurs, cytosine RNA methyltransferases (m⁵C-RMTs) form a covalent thioester bond, connecting the cysteine residue of its catalytic domain to the C6 position, thereby forming an m⁵C-RMT-RNA adduct. Next, RMT transfers a methyl group from the cofactor S-adenosyl methionine (SAM) to C5 of cytosine, followed by enzyme release from the

adduct β -elimination. 5Aza is a suicide mechanism-based inhibitor of m^5C -RMTs⁴. RNAs containing 5Aza at the precise target site will sequester the m^5C -RMT by generating RNA- m^5C -RMT adducts, which will result in the decreased level of active endogenous enzymes⁵. Consistent with this theory, we observed an obvious decreased DNMT2, rather than NSUN2 protein upon 5Aza treatment in SARS-CoV-2 infected cells (Fig. S15). It suggests that DNMT2 is more likely the main enzyme for m^5C RNA methylation in SARS-CoV-2. Further investigations are underway to elucidate the relevance of m^5C methylation and SARS-CoV-2 infection to 5Aza treatment.

In summary, we demonstrated that 5Aza can incorporate into SARS-CoV-2 RNA and disturb m^5C RNA methylation modification, potentially contributing to 5Aza's anti-SARS-CoV-2 activity. We repurposed 5Aza as a promising candidate for combating COVID-19 and introduced the possibility of targeting viral epigenomes as a novel antiviral strategy.

References

- 1 Khoddami, V. & Cairns, B. R. Identification of direct targets and modified bases of RNA cytosine methyltransferases. *Nature Biotechnology* **31**, 458-464, doi:10.1038/nbt.2566 (2013).
- 2 Dev, R. R. *et al.* Cytosine methylation by DNMT2 facilitates stability and survival of HIV-1 RNA in the host cell during infection. *Biochem J* **474**, 2009-2026, doi:10.1042/BCJ20170258 (2017).

- 155 3 Courtney, D. G. *et al.* Epitranscriptomic Addition of m(5)C to HIV-1
156 Transcripts Regulates Viral Gene Expression. *Cell Host Microbe* **26**, 217-227
157 e216, doi:10.1016/j.chom.2019.07.005 (2019).
- 158 4 Santi, D. V., Garrett, C. E. & Barr, P. J. On the mechanism of inhibition of
159 DNA-cytosine methyltransferases by cytosine analogs. *Cell* **33**, 9-10,
160 doi:10.1016/0092-8674(83)90327-6 (1983).
- 161 5 Schaefer, M., Hagemann, S., Hanna, K. & Lyko, F. Azacytidine inhibits RNA
162 methylation at DNMT2 target sites in human cancer cell lines. *Cancer Res* **69**,
163 8127-8132, doi:10.1158/0008-5472.CAN-09-0458 (2009).

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166 Acknowledgements

167 We thank Tao Du, Jin Xiong, and other colleagues from BSL-3 Laboratory of
168 Wuhan Institute of Virology and Wuhan National Biosafety Laboratory for their
169 critical support and excellent coordination. We thank Prof. Meilin Jin of
170 Huazhong Agricultural University for kindly providing mouse-adapted
171 SARS-CoV-2 strain. We thank Editage (www.editage.cn) for English language
172 editing. This work was supported by the National Science and Technology
173 Major Projects (grant number 2020ZX10001016).

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175 Competing interest

176 The authors declare no competing interests.

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178 Figure legend

179 **Fig. 1 Azacytidine targets SARS-CoV-2 RNA to inhibit virus infection.**

180 **a, b, and c** The anti-SARS-CoV-2 effect of Azacytidine *in vitro*. Vero E6 cells
181 were infected with SARS-CoV-2 at MOI = 0.2 in the presence of different

182 doses of Azacytidine. The chloroquine was used as a drug control. At 24 hpi,
183 cell supernatants were collected. **a** IC₅₀ and EC₅₀ were calculated by detecting
184 viral RNA through RT-PCR and CCK-8 assay, respectively. Left and right
185 Y-axes represent mean % inhibition of virus and % cytotoxicity of 5Aza,
186 respectively. **b** Cell supernatants were collected at 24 hpi, and the viral titers
187 were measured using the plaque assay. **c** Immunofluorescence microscopy of
188 virus infection via probing N protein of SARS-CoV-2. Bars, 200 μM. **d, e, and f**
189 The anti-SARS-CoV-2 effect of Azacytidine *in vivo*. 6–7 weeks old female
190 BALB/c mice were randomly divided into three groups with 9 mice per group.
191 Mice were intranasally challenged with 2×10^3 PFU MA-SARS-CoV-2 in 50 μl
192 DMEM (infection groups) or equal DMEM (mock infection). At 1 dpi, mice were
193 intraperitoneally injected with 2 mg/kg 5Aza (SARS-CoV-2+5Aza group) or an
194 equivalent volume of sterile saline (SARS-CoV-2+saline and mock infection
195 groups), once daily for seven consecutive doses. **d** body weight was measured
196 daily for 7 d (n=6), mice with 25% body weight loss were considered moribund
197 and euthanized. Note: the remaining one mouse (body weight loss less than
198 25%) in SARS-CoV-2+saline group was not recorded at dpi 6 and 7. **e** the
199 survival rates of mice (n=6); mice with more than 25% of body weight loss in **d**
200 were considered moribund and euthanized. **f** at 4 dpi, three mice per group
201 were euthanized for detecting viral RNA copy and virus titer in the lungs. **g** The
202 pseudo m⁵C locations indicated incorporated azacytidine. Vero-E6 cells were

infected with 1 moi SARS-CoV-2 in the presence of 10 μ M 5Aza or saline.

After 12 h, total RNA was isolated and comparison of 5Aza-treated viral RNA with the control using bisulfite sequencing was performed; the non-overlapping points are pseudo m⁵C locations that indicate where 5Aza was incorporated. **h** RNAs of cells infected with SARS-CoV-2 that treated with or without 5Aza were subjected to Nanopore direct RNA sequencing. **i** Vero E6 cells transiently overexpressing DNMT2, NSUN2, or GFP with a HA tag were infected with 0.2 moi SARS-CoV-2 for 20 h, in the presence of 16 μ M 5Aza or not. Lysates were prepared and split for incubation with mouse anti-HA antibody. Co-precipitated RNA was analyzed by qRT-PCR using primer sets targeting viral S gene. The level of viral RNA amplicon was determined as the percentage of input (1% of lysate) (right); the expression of indicated protein and products of IP was validated by western blot (left). Data are means $\pm$ SD analyzed using Student's *t* test or One-way ANOVA (body weight change); Log-rank test was used to analyze the significance of survival differences. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

224 Fig. 1